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I, RONALD MAXWELL MAY, ASSISTANT DIRECTOR PATENT ADMINISTRATION, hereby certify that the annexed are true copies of the Provisional specification and drawing(s) as filed on 19 July 1991 in connection with Application No. PK 7322 for a patent by UNIVERSITY OF QUEENSLAND filed on 19 July 1991.

I further certify that the name of the applicant has been amended to THE UNIVERSITY OF QUEENSLAND pursuant to the provisions of Section 104 of the Patents Act 1990.

I further certify that the annexed documents are not, as yet, open to public inspection.

WITNESS my hand this Twenty Fourth day of July 1992.

RONALD MAXWELL MAY
DELEGATE OF COMMISSIONER OF PATENTS

PK 7322 19 July 1991

THE UNIVERSITY OF QUEENSLAND

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION FOR THE INVENTION ENTITLED:

PAPILLOMA VIRUS VACCINE

This invention is described in the following statement:

THIS INVENTION relates to papilloma viruses and in particular antigens and vaccines that may be effective in treatment of infections caused by such viruses.

Papilloma virus infections are known not only in humans but also in animals such as sheep, dogs, cattle, coyotes, wolves, possums, deer, antelope, beaver, turtles, bears, lizards, monkeys, chimpanzees, giraffes, impala, elephants, whales, cats, pigs, gerbils, elks, yaks, dolphins, parrots, goats, rhinoceros, camels, lemmings, chamois, skunks, Tasmanian devils, badgers, lemurs, caribou, armadillo, newts and snakes (see for example "Papilloma virus Infections in Animals" by J P Sundberg which is described in Papilloma viruses and Human Disease, edited by K Syrjanen, L Gissman and L G Koss, Springer Verlag 1987).

It is also known (eg. In Papilloma viruses and Human Cancer edited by H Pfister and published by CRC Press Inc 1990) that papilloma viruses are included in several distinct groups such as human papilloma viruses (HPV) which are differentiated into types 1-56 depending upon DNA sequence homology. A clinicopathological grouping of HPV and the malignant potential of the lesions with which they are most frequently associated may be separated as follows.

In a first group may be listed types 1 and 4 which cause benign plantar warts, types 2, 26, 28 and 29 which cause benign common warts, Types 3, 10 and 27 which cause benign flat warts and Type 7 which causes butcher's warts. This first group of infections occur in normal or immunocompetent individuals.

In a second group which refer to immunocompromised individuals there may be listed Types 5 and 8 which cause highly malignant macular lesions, Types 9, 12, 14, 15, 17, 19-25, 36 and 46-50 which cause macular or flat lesions which are benign or rarely malignant. These macular lesions are otherwise known as epidermodyplasia verruci formis (EV).

In a third group there may be listed Types 6, 11, 34 and 39, 41-44, and 51-55 which cause condylomata which is rarely malignant, Types 13 and 32 which cause benign focal epithelial hyperplasia, Types 16 and 18 which cause highly malignant condylomata and possibly bowenoid papulosis and Types 30, 31, 33, 35, 45 and 56 which cause immediately malignant condylomata. The condylomata appear mostly in the anogenital tract and in particular the cervix. Types 16 and 18 are associated with the majority of in situ and invasive carcinomas. The condylomata may also occur in the aerodigestive tract.

The animal papilloma viruses may also include bovine papilloma virus (BPV) and in particular types BPV1, BPV2, BPV3, BPV4, BPV5 and BPV6 which are also differentiated by DNA sequence homology. In general the other animal papilloma viruses infect deer, horses, rabbits, dogs, rodents and birds.

Papilloma viruses are small DNA viruses encoding for up to eight early and two late genes. (for review see Lancaster and Jenson 1987 Cancer Metast. Rev. p6653-6664; and Pfister 1987 Adv. Cancer Res 48, 113-147). The organisation of the late genes is simpler than the early genes. The late genes L1 and L2 slightly overlap each other in most cases.

The putative L2 proteins are highly conserved among different papilloma viruses particularly the sequence of 10 basic amino acids at the C-terminal end. The broad domain in the middle reveals only small clustered similarities. The L1 ORF however appears monotonously conserved in all known cases. (See Syrjanen et al above). The amino acid sequence homology reaches 50% with the comparison between HPV1a, HPV6b, BPV1 and CRPV (Cotton tail rabbit papilloma virus).

In regard to immunotherapy concerning papilloma virus infections prior methods of treatment of warts and epithelial skin lesions have involved the use of surgery which can be painful and traumatic with scarring often a result with the risk that reinfection can occur. Treatment with chemicals has also been used. A common treatment agent is salicylic acid which is the main ingredient in strengths ranging from 10% to 40% in tinctures and plasters. Formalin in strengths of 3% - 20% has also been proposed. Cryotherapy has been used for treatment of skin warts. Gluteraldehyde as a treatment agent has also been used. Podophyllin has also been used with varying success for both skin warts and anogenital condylomata. The types of surgery that has been used on anogenital condylomata has included surgical excision, cryosurgery and laser surgery. The use of interferons has also been proposed (see Syrjanen et al above).

Vaccines have also been proposed with indifferent success. It has been proposed to use vaccines containing autogenous tumor homogenates [Abcarian et al J. Surg Res 22: 231-236 (1977) Dis Colon Rectum 25:648-51 (1982) Dis Colon

Rectum 19: 237-244 (1976)]. However it has recently been advocated that patients should no longer be treated with autogenous vaccines because of the potential oncogenic effect of the viral DNA (Bunney 1986 Br Med J 293 1045-1047).

In relation to production of genetically engineered vaccines this matter has been discussed in Pfister (1990) above and it seems that difficulty has been experienced in obtaining an effective vaccine because of the plethora of different papilloma virus types. Pfister however points out that attention should be directed to the so called early proteins (ie. E1, E2, E3, E4, E5, E6, E7 or E8) because these proteins are most likely synthesised in the proliferating basal cells of a wart infection in contrast to the structural proteins which are expressed in the upper epidermal layers. Therefore according to Pfister (1990) virus capsid protein appears to be limited in relation to use in a vaccine. The use of recombinant vaccinia viruses in <u>in vitro</u> test systems for papilloma virus early proteins in eukaryotic cells has been discussed also in Pfister (1990). This may take the form of a live vaccine consisting of genetically modified vaccina virus expressing papilloma virus proteins or on the surface of paraformaldehyde fixed autologous cells infected <u>in vitro</u> with vaccinia recombinants or transfected with other expression vectors. Another strategy for vaccine development as discussed in Pfister (1990) is to use an immune stimulating complex of the glycoside Quil A.

Data on successful proplylactic vaccination exist only for bovine fibropapillomas homogenised homogenate of

bovine fibropapillomas and has been shown to provide limited immunity (Olson et al J Am Vet Med Assoc 135, 499 (1959) Cancer Res 22 463 (1962)). A vaccine including an engineered L1 fusion protein (Pilacinski et al. UCLA Symp. Molecular and Cellular Biology New Series Vol 32 papilloma Viruses Molecular and Clinical Aspects Alan R Liss New York 1985 257) has also been used in calves but proved unsuccessful in humans (Barthold et al J. Am Vet Med Assoc. 165, 276, 1974). In Pfister (1990) it is stated that there is presently no evidence for a possible prevention of HPV infection by the use of a capsid protein vaccine, but induction of an antitumor cell immunity appears to be feasible.

The L1 and L2 genes have been the basis of vaccines for the prevention and treatment of papilloma virus infections and immunogens used in the diagnosis and detection of papilloma viruses (International Patent Specifications WO8605816 and WO8303623). However, it appears that no commercial usage of these vaccines have taken place.

Therefore it is an object of the invention to provide a vaccine for use with papilloma virus infections which is effective in use.

The invention therefore in one aspect includes a method for production of a vaccine which includes the steps of:

(i) constructing one or more recombinant DNA molecules encoding the L1 and L2 proteins;

(ii) transfecting a suitable host cell with said one or more recombinant DNA molecules so that virus like particles

(VLPs) are produced within the cell after expression of the L1 and L2 proteins; and

(iii) obtaining the VLPs from the transfected cells and incorporating the VLPs in a vaccine.

The invention also includes within its scope the VLPs per se as well as their use as an antigen for diagnostic pruposes for example.

In relation to step (i) it will be appreciated that the L1 and L2 genes may be included in the same DNA recombinant molecule or in different DNA recombinant molecules.

Suitable DNA recombinant molecules include plasmids, cosmids, baculovirus, vaccinia virus adenovirus or retrovirus. These molecules may be used to transfect a suitable prokaryote host cell such as E. coli or eukaryote cell such as yeast, an insect cell such as S. frugiperda or mammalian cell. Suitable expression systems may include the following:

(i) Prokaryotic systems including E. coli and any plasmid expression vector

(ii) Eukaryote systems including yeast and yeast plasmids, S. frugiperda and recombinat baculovirus, any eukaryote cell and recombinant vaccinia, adenovirus or retroviruses and any eukaryote cell with the L1 and L2 gene driven off any mammalian or viral promoter with a mammalian or viral polyadenylation signal.

In the experimental work that has taken place the L1 gene is located downstream of a vaccinia 4b promoter and the L2 gene is located downstream of a synthetic vaccinia 28k late

promoter. The host cell is monkey epithelial cells.

The VLPs may be obtained from the transfected cells by any suitable means of purification. The VLPs may be combined with any suitable adjuvant such as alum, Freunds Incomplete or Complete Adjuvant, Quil A and other saponins or any other adjuvant as described for example in Vanselow (1987) S. Vet. Bull. 57 881-896.

MATERIAL AND METHODS

Recombinant Vaccinia Viruses

The HPV-16 L1 gene, from the second ATG (nt5637), was amplified by polymerase chain reaction from pHPV16(provied by Dr. Gissmann), using following primers:

1/ 5'-CAGATCTATGTCTCTTTGGCTGCCTAGTGAGGCC-3'

2/ 5'-CAGATCTAATCAGCTTACGTTTTTTGCGTTTAGC-3'

The first methionine codon and stop codon are indicated by underline, and BglII sites were included to facilitate subcloning. The amplified 1527 bp fragment was extracted with phenol and purified by 1% agarose gel electrophoresis. After digestion with BglII the L1 gene was subconed into the BamHI site of the RK19 plasmid (Kent 1988 Ph.D. thesis, University of Cambridge) which contains a strong vaccinia virus promoter (4b). The resulting plasmid was sequenced (Sanger et al, 1977, Proc. Natl. Acad. Sci. USA 74,5463-5467) and used to prepare a fragment containing the HPV16 L1 gene linked to the 4b promoter by digestion with MluI and SstI. This fragment was blunted with T4 DNA polymerase and cloned into the Bam HI site of the vaccinia intermediate vector pLC1, which contains the B24R gene of vaccinia virus

(Kotwal and Moss, 1989, J. Virol. 63, 600-606; Smith et al, 1989, J. Gen. Virol. 70, 2333-2343), an E. coli gpt gene (Falkner and Moss, 1988, J. Virol. 64, 1849-1854; Boyle and Coupar, 1988, Gene 65, 123 - 128) and multiple cloning sites to produce plasmid pLC200.

The HPV16 L2 gene was prepared by partial digestion of pHPV16 with Accl to produce a fragment (4138nt-5668nt) which was filled with Klenow and linked to synthetic BamHl linkers. This L2 fragment was cloned into the Bam HI site of a pUC derived plasmid termed p480 which has a synthetic vaccinia 28K late promoter, with some modifications (Davison and Moss, 1989, J. Mol. Biol. 210, 771-784). The promoter sequence is as follows:

5'-GAGCTCTTTTTTTTTTTTTTTTTTTTGGCATATAAATGGAGGTACCC-3'

the late promoter motif is underlined. A fragment containing the L2 gene linked to the 28K promoter was isolated by digestion with SstI/SalI, blunted by T4 DNA polymerase and then cloned into the SstI and SalI sites of pLC200 to produce pLC201(Fig.1).

An HPV16 E1/E4 cDNA clone (Doorbar et al, 1990, Viorl. 178, 254-262) prepared from W12 cell cDNA (Stanley et al, 1989, Int. J. Cancer 43, 672-676), comprising the first 16 bp of the HPV16 E1 gene spliced into the E4 region at 3357nt, was cloned into the p11K plasmid (Zhou et al, 1991a, Virol 181,203-210) which has a synthetic vaccinia virus 11K late promoter. A fragment containing the E1/E4 gene linked to the 11K promoter was isolated by EcoRI/Xbal digestion, blunted with Klenow and cloned into pLC201 to produce pLC202 (Fig. 1).

The pLC201 and pLC202 plasmids were then used to construct recombinant vaccinia viruses as previously described (Mackett et al, 1984, J. Virol. 49, 857-864). Recombinant viruses pLC201VV and pLC202VV were selected by plaque assay in the presence of mycophenolic acid, xanthine, and hypoxanthine (Falkner and Moss, 1988). Recombinant VV expressing HPV16L1, and HPV16L2, were prepared and used as previously described (Zhou et al, 1990, J. Gen. Virol. 71, 2185-2190).

PURIFICATION OF VIRUS-LIKE PARTICLES

CV-1 cells infected with recombinant viruses pLC201VV or pLC202VV were harvested in 10 mM Tris(pH 9.0) 32 hr after infection and homogenised with a Dounce homogeniser. Homogenates were clarified by centrifugation at 2000g to remove the cell debris and layered onto a 30% (wt/vol) sucrose cushion. The pellet formed by centrifugation at 110,000g in a SW38 rotor for 90 min was suspended in 10 mM Tris pH9.0 and layered onto a 20-60% discontinuous sucrose gradient. After centrifugation at 100,000g for 18 hrs, 10 equal fractions of 0.25 ml were collected. Samples were mixed with 0.6 ml ethanol. The pellet obtained after centrifugation at 4°C and 12000g for 20 minutes was collected for further analysis. To determine the density of the virus-like particles, equilibrium density-gradient sedimentation was accomplished in CsCl (1.30g/ml). After centrifugation at 125,000 xg for 20 hrs, 11 fractions of 0.25ml were collected. The density of each fraction was determined, and each was examined for virus-like particles by transmission electron microscopy.

ELECTRON MICROSCOPY

CV-1 cells infected with recombinant vaccinia virus were fixed in 3% (vol/vol) glutaraldehyde in 0.1M sodium cacodylate buffer and postfixed in 1% osmium tetroxide, dehydrated in graded alcohols, and embedded in epoxy resin. Thin section were cut and stained with uranyl acetate and lead citrate. Fractions from the sucrose gradient were dried onto EM grids, and negatively stained with 1% (wt/vol) phosphotungstic acid (pH7.0). Fractions were examined using a JEOL 1200Ex Transmission electron microscope.

ANALYSIS OF HPV ORF PRODUCTS

HPV16 L1 and E1/E4 gene expression was analysed by immunoprecipitation and immunoblot. For immunoprecipitation, ^{35}S metabolically labelled recombinant VV infected CV-1 cells were lysed in RIPA buffer 0.1% SDS, 1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 0.5 μg/ml aprotinin, 10mM Tris-HCl, pH7.4). Immunoprecipitation with the E1/E4 specific monoclonal antibody (MAb) 3F7 was performed as previously described (Zhou et al, 1991a, Virol. 181, 203-210). Immunoblot analysis of partially purified virus-like particles, using the L1 specific MAb Camvir1 (Mclean et al, 1990, J. Clin. Pathol. 43, 488-492) and ^{125}I anti-mouse IgG(Amersham), was performed as previously described using samples solublised in 2x SDS gel loading buffer containing 2-mercaptoethanol. For analysis of HPV16 L2 gene expression, for which no specific antiserum was available, RNA blotting was performed using RNA prepared from CV-1 cells infected with 30 pfu/cell virus for 12 hrs. Virus RNA was purified as

described by Cooper and Moss (1979, Virol. 96, 368-380), separated (20μg/track) on 1.2% formaldehyde gels (Maniatis et al, 1982, Cold Spring Harbor laboratory, Cold Spring Harbor), transfered to nylon membranes, and probed with ^{32}P-labelled L2 fragment. For analysis of N-glycosylation, partially purified virus-like particles were taken up to 100 μl buffer (0.25 M sodium acetate, pH6.5, 20 mM EDTA and 10 mM 2-mercaptoethanol) and reacted with 0.5 u Endoglycosidase F (Boehringer Mannheim) at 37°C for 18 hrs prior to immunoblotting.

RESULTS

Mycophenolic acid was used to select vaccinia viruses recombinant for the gpt plasmids pLC201 and pLC202, and these were termed pLC201VV and pLC202VV. Synthesis of L1 in cells infected with pLC201VV, and L1 and E1/E4 in pLC202VV infected cells, was confirmed by immunoblotting and immunoprecipation. L1 protein was demonstrated as a band on autoradiography of approximately 57kDa, while E1/E4 was identified as an 11kDa band (Fig.2). A northern blot of RNA extracted from CV-1 cells infected with these recombinant viruses confirmed high levels of L2 mRNA transcription in cells infected with either of these viruses (Fig 3). L2 transcription from a synthetic vaccinia virus late promoter gave a heterogeneous Northern blot pattern because VV late RNAs do not use a specific transcription termination signal.

CV-1 cells were infected with pLC201VV and examined for virus-like particles. Electron micrographs of thin sections of cells infected with pLC201VV, but not of control cells infected only with wild-type vaccinia, showed

approximately 40nm virus-like particles in cell nuclei. In most cases these particles were linked in chains, and near the nuclear membrane (Fig. 4a). Cells infected with recombinant vaccinia viruses which expressed HPV16 L1 only or L2 only, and produced the corresponding protein (L1) or mRNA (L2), did not contain virus-like particles. Cells simultaneously infected with two different recombinant vaccinia viruses, which expressed HPV16 L1 and HPV16 L2 respectively, also failed to make any HPV virus-like particles; although L1 protein and L2 mRNA could be identified in pools of these double infected cells simultaneous synthesis of both L1 and L2 within individual cells was not demonstrated.

To confirm that the virus-like particles observed by electron microscopy contained HPV16 L1 protein, cell extracts from pLC201VV infected cells were subjected to a partial purification in a 20%-60% sucrose gradient. Ten fractions were collected and examined for L1 protein. From fractions 3 to 7, L1 could be detected and in fraction 5, the highest level of L1 was found. Each fraction was also examined by EM for virus-like particles: these were observed in fraction 5. A typical papilloma virus negatively-stained with sodium phosphotungstate, has 72 regular close-packed capsomeres (Finch and Klug, 1965, J. Mol. Biol. 13, 1-12; Rowson and Mahy, 1967, Bacteriol. Rev. 31, 110-131) and has a diameter about 50nm. The diameter of the virus-like particles purified from the infected CV-1 cells varied between 35nm and 40nm. These virus-like particles however possessed a similar EM appearance to papilloma viruses, and a regular array of

capsomeres could be recognised (Fig 4b). The virus-like particles identified in fraction 5 of the sucrose gradient were therefore presumed to be empty and incorrectly assembled arrays of HPV capsomeres. In CsCl, HPV16 virus-like particles sedimentated at about 1.31 g/ml(Fig 5), and showed a typical empty papilloma virus capsid appearance under transmission electromicroscope (Fig 5, insert).

These data suggest that the E4 protein, held to play a role in papilloma virus assembly does not appear to be an essential element for capsomere production and assembly in vivo. To test whether E4 could enhance production of virus-like particles in our system, CV-1 cells were infected with the HPV16 L1, L2 and E1/4 triple recombinant virus pLC202VV. Virus-like particles similar to those seen in cells infected with pLC201VV were produced, but no quantitive or qualitative differences in the virus-like particles were observed to support a role for the E1/4 protein in capsomere assembly. This conclusion is supported by the observation that E1/E4 protein, as demonstrated by immunofluorescence, remains in the cytoplasm of E1/E4 recombinant vaccinia virus infected cells while the structural proteins L1 and L2, which contain a nuclear targeting signal move from the cytoplasm to the nucleus, where the capsomeres are produced and virions assembled.

Camvir-1 identified a protein doublet in western blots of virus-like particles purified from pLC201VV infected CV-1 cells (Fig 6). HPV16 L1 contains four potential N-glycosylation sites (asparagine 157, 242, 367 and 421). To

test whether the doublet represented glycosylation variants of the L1 polypeptide, partially purified virus-like particles were subjected to treatment with endoglycosidase F, prior to SDS-PAGE and immunoblotting. This resulted in the replacement of the doublet by a single band of slightly lower apparent molecular weight, at the expected molecular weight of about 57 kDa (Fig 6. lane 2,3).

DISCUSSION

Papilloma viruses generally produce virions in infected keratinocytes which are readily identifiable by electron microscopy (Almeida et al, 1962, J. Invest, Dermatol 38, 337-345) and which in some cases can be purified and shown to be infectious (Rowson and Mahy, 1967, Bacterial. Reo. 31, 110-131). HPV 16 virions are however, not seen in HPV16 infected cervical epithelial tissue although HPV16 L1 and L2 late gene transcription occurs in differentiated genital epithelium (Crum et al, 1988. J. Virol. 62, 84-90) and L1 translation produces immunoreactive L1 protein in these tissues (Stanley et al 1989, Int. J. Cancer 43, 672-676). In this study we have shown that expression of HPV16 L1 and L2 genes in epithelial cells is both necessary and sufficient to allow assembly of virion-like particles and thus the L1 and L2 proteins of HPV 16 are not defective with regard to virion assembly. The expression of HPV16 late genes in tissues appears to be strictly regulated by the epithelial environment (Taichman et al, 1983, J. Invest. Dermatol 1, 137-140). Failure to detect HPV16 virions <u>in vivo</u>, despite transcription of L1 and L2 and translation of L1, suggests that there is

either a post transcriptional block to L2 production in cervical epithelium, or an inhibitor of virion assembly. In the HPV16 containing cell line W12, derived from cervical tissue, virus-like particles were observed when the cells underwent terminal differention in vivo in a murine microenvironment (Sterling et al 1990. J. Virol 64, 6305-6307) suggesting that such cells have no block to virion assembly, and that insufficient translation of L2 or other unknown reasons may explain failure to demonstrate HPV16 virions in cervical tissues.

In cervical intraepithelial neoplasia, HPV16 E4 expression is observed in foci of cells also expressing L1 (Crum et al, 1990, Virol. 178, 238-246). While the function of E4 is unknown, its relative abundance (Breitburd et al, 1987, Cold Spring Harbor laboratories, Cold Spring Harbor) in HPV infected cells and the tight linkage with keratinocyte differentiation and HPV late protein expression has allowed speculation that it plays a role in virion assembly (Doorbar et al, 1986, EMBO J 5, 355-362; Doorbar et al, 1989, Virol. 172, 51-62). E4 is a cytoplasmic protein (Doorbar et al 1986) while L1 and L2 contain a nuclear targeting signal and move rapidly from cytoplasm to nucleus where they are presumably assembled into virus particles. These and our data suggest that the E4 protein of HPV16 is not a structural component of the HPV virion and does not appear to be essential for virion assembly.

Our EM studies show that the empty HPV16 virion has an average size of about 40 nm which is smaller than other

papilloma viruses, but has a similar surface structure compared with other papilloma viruses such as rabbit papilloma virus (Finch and Klug, 1965), or human wart virus (Rowson and Mahy 1967). Sedimentation showed an empty capsid density of about 1.31g/ml, the density expected of empty papilloma virus capside compared with about 1.36g/ml for intact HPV1a virions (Doorbar and Gallimore, 1987, J. Virol. 61, 2793-2799).

The L1 protein from HPV has potential glycosylation sites, and purified BPV particles have minor electrophoretic forms of L1 whose mobility is sensitive to endoglycosidase treatment (Larsen et al, 1987, J. Virol 61, 3596-3601). L2 from HPV 1a and HPV 11 has been observed to be a doublet (Rose et al, 1990, J. Gen. Virol, 71, 2725-2729; Doorbar and Gallimore, 1987; Jin et al, 1989, J. Gen. Virol. 70, 1133-1140) and this has been attributed to differences in glycosylation. Our data show that the L1 protein in HPV16 capsomeres is also glycosylated, and that two different glycosylation states exist. Production of virion-like particles in our system should allow study of the role of specific receptors in the binding and uptake of HPV16 by epithelial cells, and of the role of glycosylation of L1 in this process.

LEGEND

Fig 1 Plasmids used to construct recombinant vaccinia viruses. HPV16 L1, L2 and E1/E4 genes (open boxes) are under control of vaccinia late promoters (solid boxes). E. coli gpt gene (shaded box) is used as selection marker. Flanking sequence for homologous recombination. The direction

of transcription is indicated by arrows.

Fig 2 Analysis of HPV16 L1 and E1/E4 proteins expressed by recombinant vaccinia viruses.

A. Western blot analysis of recombinant HPV16 L1 in vaccinia virus infected CV-1 cells. Cells were infected at 10 pfu/cell with wt VV (lane 1), pLC201VV (lane 2) or pLC202VV (lane 3) and harvested 48h post infection. L1 proteins was detected with the HPV16 L1 specific MAb Camvir 1. The 57kDa L1 protein is indicated by the arrow. Binding of Camvir1 to the 35 kDa protein in all three lanes is non-specific.

B. Immunoprecipitation analysis of E1/E4 from vaccinia virus infected CV-1 cells. Cells infected with 10 pfu/cell with pLC202VV(lane 1), pLC201VV(lane 2) or wt VV (lane 3) were labelled with ^{35}S-methionine. The E1/E4 protein was immunoprecipitated with an HPV16 E4 specific MAb 3F7. The 11kDa E1/E4 band is indicated by arrow. At the left, the position of protein size markers is shown.

Fig 3 Northern blot analysis of recombinant vaccinia virus infected CV-1 cells. RNAs extracted from cells infected with pLC201VV (lane 2), pLC202VV(lane 3) or wt VV (lane 4) were resolved on a 1.2% formaldehyde-agarose gel. RNA was transfered to nylon membrane and hybridised with a ^{32}P-labelled L2 probe. Formaldehyde-treated lambda DNA-Hind III cut marker are shown (lane 1).

Fig 4 Electron microscopy of HPV virus-like particles from CV-1 cells infected with recombinant vaccinia virus.

A, CV-1 cells infected with pLC201VV for 32 hours were fixed

in 3% glutaraldehyde and postfixed in 1% osmium tetroxide. The specimen was then resin embedded, section and uranyl acetate stained, using standard protocols. In the CV-1 nuclei, particles of approximately 40-nm diameter (arrowed) were frequently found. The bar corresponds to 100 nm.

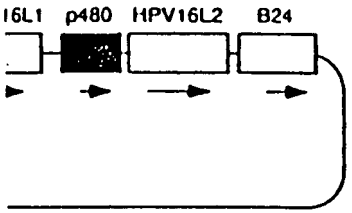
B, Fraction 5 of the sucrose gradient was negatively stained with 1% phosphotungstic acid (pH7.0). Papilloma virus-like particles, apparently consisting of regular arrays of capsomeres were observed (arrowed). The bar corresponds to 50nm.

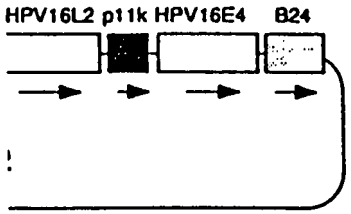
Fig 5 CsCl equilibrium density-gradient sedimentation of HPV16 empty capsid. HPV16 virus-like particles obtained from CV-1 cells infected with pLC201VV were centrifuged over a sucrose cushion and then subjected to CsCl isopynic sedimentation. Virus-like particles (+) were found in fractions 8 and 9. The transmission electron micrograph of a negatively stained particle from fraction 8 is shown in the insert. The density (g/ml) of each gradient fraction is indicated.

Fig 6 Glycosylation of L1 proteins in purified virus particles. CV-1 cells were infected with pLC201VV for 32 hrs, and virus-like particles were purified on a sucrose gradient. Samples were precipitated with ethanol and treated with endoglycosidase F before analysis by immunoblotting with an anti-HPV16 L1 antibody Camvir I. Lane 1: purified virus-like particles; Lane 2 and 3: after treatment with endoglycosidase F overnight. The L1 doublet is indicated by (=), and deglycosylated L1 is indicated by the arrow. Molecular weight markers are shown on the left.

Fig 7 Flow diagram of the construction of pLC200 encoding L1 and pLC201 encoding L1 and L2.

Fig 8 Flow diagram of the construction of pLC202 encoding L1, L2 and E1/E4.





1.

Fig 2A.

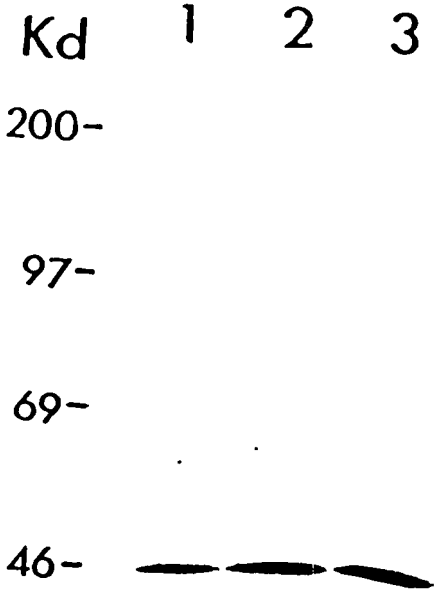
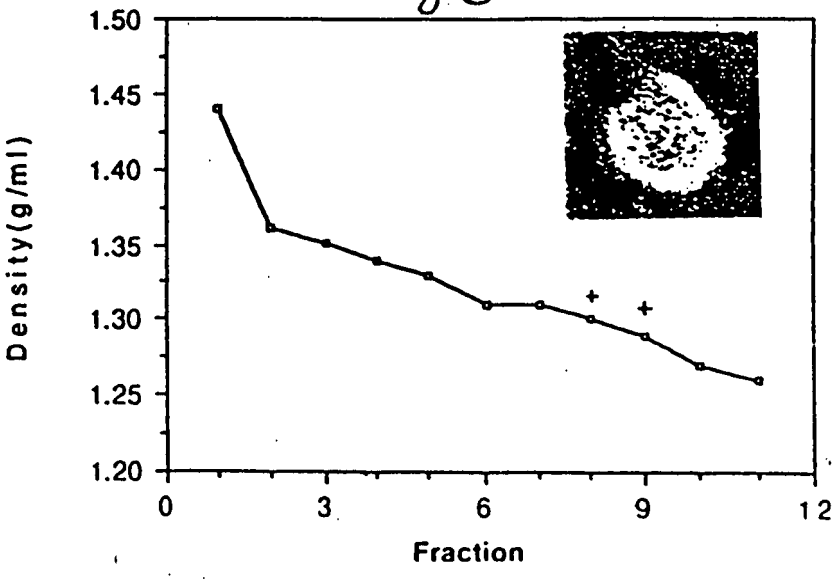


Fig 6

Fig 5.



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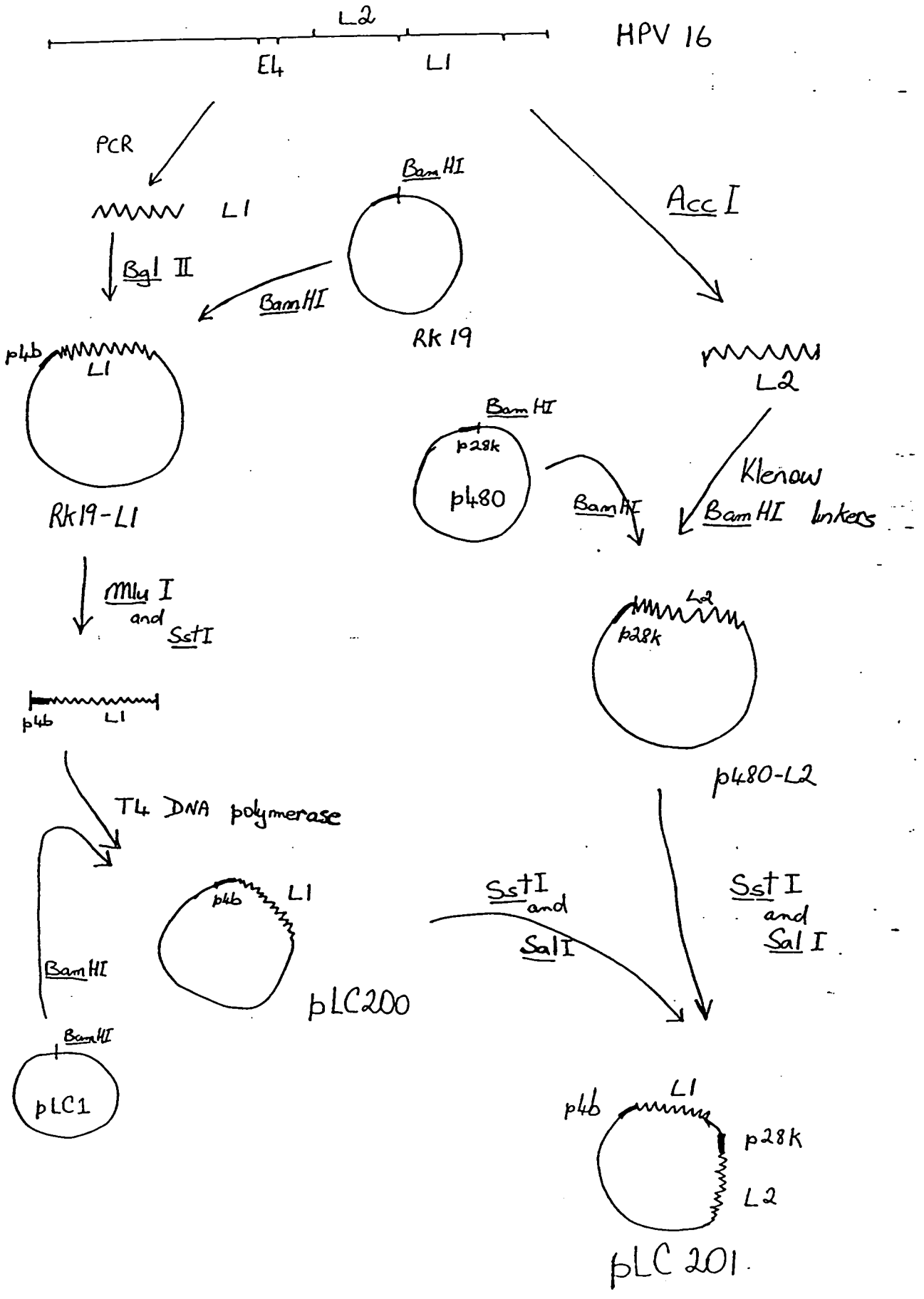
L2
E4
L1
HPV 16
PCR
L1
Bgl II
BamHI
BamHI
Rk 19
Acc I
p4b
L1
Rk19-L1
BamHI
p28k
p480
BamHI
L2
Klenow
BamHI linkers
Mlu I
and
SstI
p4b
L1
L2
p28k
p480-L2
T4 DNA polymerase
p4b
L1
BamHI
pLC200
SstI
and
SalI
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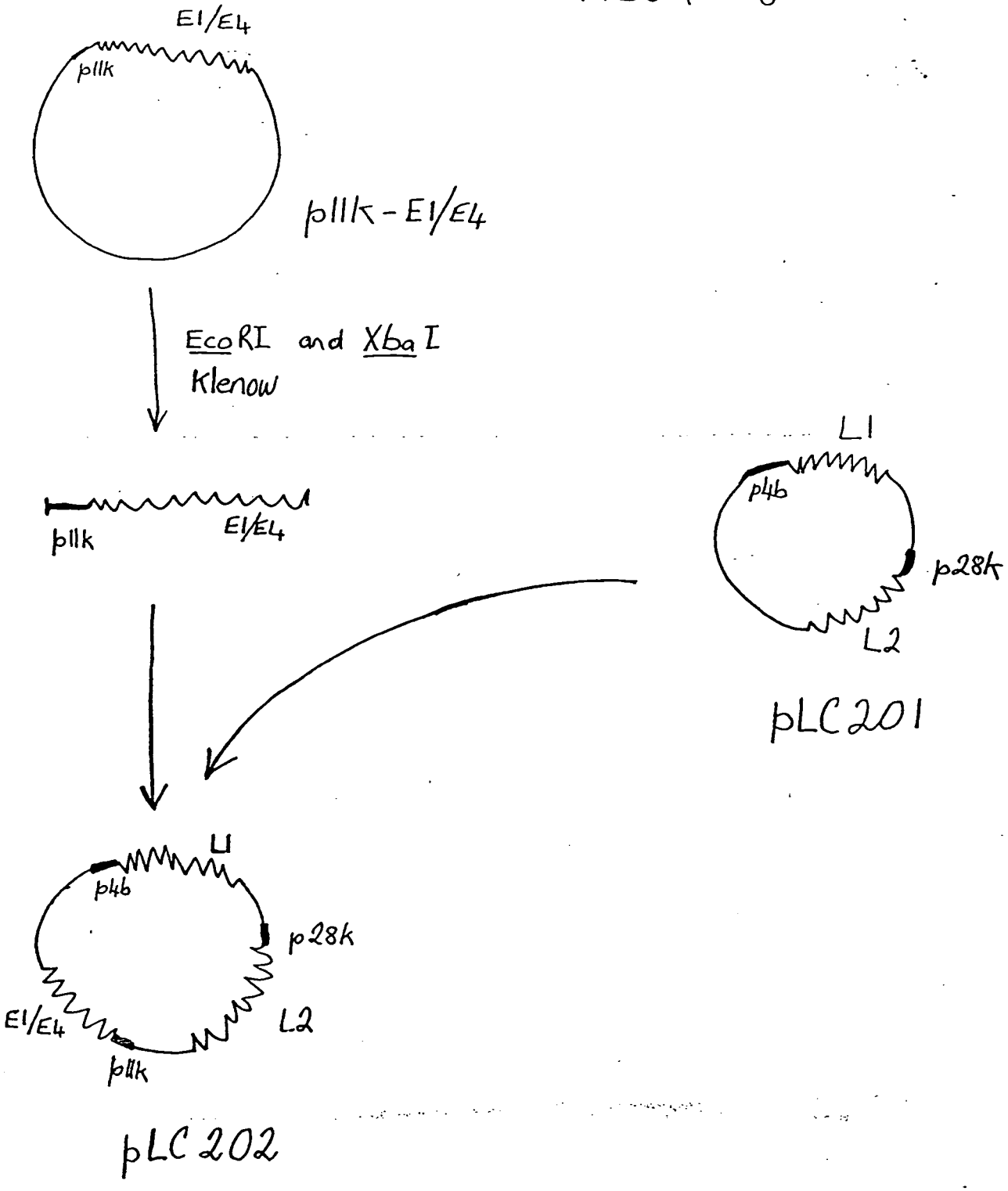
and
SalI
BamHI
pLC1
p4b
L1
p28K
L2
pLC 201

FIGURE 8


E1/E4
pIIk
pIIk - E1/E4
EcoRI and XbaI
Klenow
pIIk
E1/E4
L1
p4b
p28k
L2
pLC201
L1
p4b
p28k
L2
E1/E4
pIIk
pLC202

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